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<b>(54) Title:</b> METHODS AND NUCLEIC IMMUNOGENIC COMPOSITIONS ENCODING ANTIGENS AND CO-STIMULATORY MOLECULES FOR IMMUNIZATION			
<b>(57) Abstract</b> <p>Nucleotide vectors encoding antigens and co-stimulatory molecules, such as B7-1 (CD80) or B7-2 (CD86), are described. These vectors may be administered to a host by a variety of routes to evoke an immune response. Antigens include those from pathogens and tumour-associated antigens. In particular, the immune response may be a cytotoxic T cell response and, if the antigen is from a pathogen, may provide protection against disease caused by the pathogen. To further augment the immune response, a gene encoding a cytokine, such as granulocyte-macrophage stimulating factor (GM-CSF) and interleukin-12 (IL-12), may also be administered. The vectors have utility in vaccination and therapy of infectious or neoplastic disease.</p>			

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METHODS AND NUCLEIC IMMUNOGENIC COMPOSITIONS  
ENCODING ANTIGENS AND CO-STIMULATORY  
MOLECULES FOR IMMUNIZATION

5

FIELD OF INVENTION

The present invention relates to the field of immunology and is particularly concerned with immunogenic compositions comprising nucleic acid molecules encoding antigens, co-stimulatory molecules and, optionally, cytokines.

REFERENCE TO RELATED APPLICATION

This patent application is a continuation-in-part of copending United States Patent Application No. 08/612,553 filed March 8, 1996.

BACKGROUND OF THE INVENTION

DNA immunization has recently emerged as a promising new vaccine strategy (reviewed in (ref. 1 - throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure)). Various demonstrations that pathogen-neutralizing immune responses can be induced by the injection of purified expression plasmids encoding different non-self antigens serve to emphasize the potential of this new approach to immunization (ref. 2). Although results have indicated that both B- and T-cell immune responses can be produced by plasmid DNA immunization, less attention has been paid to the induction of cytotoxic T-lymphocytes (CTL). CTL responses are known to play an important role in the clearance of virus infection (ref. 3), and also the

development of beneficial anti-tumour responses (ref. 4). Because CTL responses are difficult to induce in the absence of an active infection, the ability to generate CTL responses via a non-infectious route, such as nucleic acid immunization, offers the potential to develop important new therapeutic and prophylactic immunogens.

Although nucleic acid immunizations have been used to generate both antibody and T-cell immune responses specific for the encoded antigens, the mechanism by which the intramuscular injection of a bacterial expression plasmid results in the priming of this immune response remains unclear (ref. 1). Intramuscular injection of plasmids containing histologically detectable reporter gene products has confirmed that gene expression can occur within target muscle fibres (ref. 5). However, it remains to be established whether or not transfected muscle cells represent the key cells presenting antigen to T-cells. Muscle cells express low levels of the class I major histocompatibility complex (MHC) gene products, and apparently lack the co-stimulatory molecules required to initiate productive T-cell activation (ref. 6). Further, the minimal inflammatory response associated with the injection of plasmid DNA in saline is unlikely to induce the cytokines normally associated with the generation of a strong T-cell response. For these reasons, there is the possibility that the key T cell induction events result from the transfection of professional antigen presenting cells (APC) either in the vicinity of or remote from the muscle injection site (ref. 7). Alternatively, protein products of the transfected gene could be released from muscle cells and mediate T-cell activation as a result of processing by physically remote APCs.

The co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) are expressed on activated B-cells and other

APCs, and their interactions with either CD28 or CTLA-4 on T-cells provide the obligatory second signal required for MHC restricted T-lymphocyte activation (reviewed in ref. 8). B7-2 is expressed before B7-1 in a developing immune response and it has been postulated that these two co-stimulatory molecules may have distinct functional features (ref. 9). In support of this concept, recent studies have shown that the two molecules can have differential effects on promoting specific T-cell effector functions (ref. 10).

IL-12 promotes CTL activity and is a 70 kD heterodimeric cytokine comprised of p35 and p40. It is most commonly produced by cells of the macrophage/monocyte lineage (reviewed in ref. 11). IL-12 induces cytokine secretion in T lymphocytes and natural killer (NK) cells, increases the cytotoxicity of CTL and NK cells, and promotes the generation of CTL (ref. 12). Since IL-12 primes CD4<sup>+</sup> T cells to produce high levels of  $\gamma$  interferon (IFN $\gamma$ ), it is considered to be a primary determinant of T<sub>H</sub>1-associated immune responses (ref. 13). The hematopoietic growth factor GM-CSF, known to stimulate the proliferation and maturation of APCs (ref. 14), has been linked to augmentation of both anti-tumour and anti-viral immune responses. Enhanced anti-tumour activity was observed when the GM-CSF gene was transduced into tumour cell immunogens (ref. 15), and also when injected as fusion protein, consisting of GM-CSF and a tumour-specific immunoglobulin idiotype (ref. 16).

The utility of administering nucleic acid molecules encoding antigens to a host to evoke immune responses, including protective immune responses, has been shown in a number of relevant test systems. The immune response generated may not be optimal for all encoded antigens however, it would be useful to provide immunogenic compositions comprising nucleic acid molecules encoding

antigens and co-stimulatory molecules and methods for their use for producing an enhanced immune response in a host to the antigen for the production of immunogenic compositions, including vaccines, and diagnostic reagents.

#### SUMMARY OF THE INVENTION

The present invention is directed towards the provision of nucleic acid molecules encoding antigens and co-stimulatory molecules and methods of their administration for producing immune responses.

In accordance with one aspect of the invention, there is provided a nucleotide vector comprising a first portion having a sequence encoding at least one antigen, a second portion having a sequence encoding at least one co-stimulatory molecule and a promoter operatively coupled to each of said first and second portions for expression of said at least one antigen and said at least one co-stimulatory molecule, including B7-1 and B7-2.

The antigen may be an antigen from a pathogen and may be selected from the group consisting of viruses, bacteria and parasites. In a particular embodiment, the pathogen may be an influenza virus and the antigen may be selected from the group consisting of structural and non-structural influenza virus antigens including haemagglutinin, neuraminidase, nucleoprotein, NS1 and NS2. In a further aspect of the invention, the antigen may be a tumour-associated antigen including carcinoembryonic antigen (CEA), mutated tumor suppressor genes, such as p53, mutated oncogenes, such as ras, or idiotypic markers for tumors, such as B-cell lymphoma.

The vector may further comprise a third portion having a sequence encoding at least one cytokine, such as granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12), interleukin-4 (IL-4)



and fragments thereof retaining cytokine activity. The vectors of the present invention may be formulated as vaccines for in vivo administration to a host to protect said host against disease caused by said pathogen.

5 In a particular embodiment there is provided a composition comprising the vector described herein and a second nucleotide vector comprising a first portion having a sequence encoding at least one cytokine and a promoter operatively coupled to said first portion of  
10 said second vector for expression of said cytokine. The cytokine may be granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-12, interleukin-4 and fragments thereof retaining cytokine activity.

15 The invention also extends in a particular aspect to a method of generating an immune response in a host comprising administering to the host an effective amount of a first nucleic acid molecule encoding at least one antigen, a second portion nucleic acid molecule encoding  
20 at least one co-stimulatory molecule and a promoter operatively coupled to each of said first and second nucleic acid molecules for expression of said at least one antigen and said at least one co-stimulatory molecule. The first nucleic acid molecule, the second  
25 nucleic acid molecule and the promoter preferably are combined in a single nucleotide vector. The antigen may be an antigen from a pathogen (including bacteria, viruses and parasites) or a tumour-associated antigen.

The immune response may be a cytotoxic T-cell immune  
30 response, and may confer protection to the host against disease caused by the pathogen or a therapeutic immune response against tumour cells.

Vectors of the present invention may be administered intraperitoneally, intravenously,  
35 subcutaneously, intramuscularly, intradermally or to a mucosal surface of a host, such as by intranasal

administration. The method of generating an immune response may further comprise the step of administering to the host a third nucleic acid molecule encoding at least one cytokine and a promoter operatively coupled to the third nucleic acid molecule gene for expression of the at least one cytokine. The cytokine may be macrophage colony stimulating factor (GM-CSF), interleukin-12, interleukin-4 and fragments thereof retaining cytokine activity. In a particular embodiment, the cytokine gene may be contained within the same vector as the genes encoding the antigens and the co-stimulatory molecules. In an alternative embodiment, the at least one third nucleic acid molecule encoding the at least one cytokine may be contained within a second nucleotide vector.

The invention extends to a method of using a first gene encoding an antigen and a second gene encoding a co-stimulatory molecule to produce an immune response in a host, comprising the steps of constructing a nucleotide vector comprising the first and second genes, operatively coupling to each of said first and second genes a control sequence to direct expression of the first and second genes in the host, and introducing the vector into the host.

The present invention also includes a method for producing an immunogenic composition for evoking a specific immune response to an antigen in a host to which the immunogenic composition is administered, comprising the steps of constructing a nucleotide vector comprising a first gene encoding the antigen and a second gene encoding a co-stimulatory molecule, operatively coupling to each of said first and second genes a control sequence to direct expression of said first and second genes in said host, and formulating said vector as the immunogenic composition for in vivo administration to the host.

The antigen may be an antigen of a pathogen and the immunogenic composition may then be formulated as a vaccine for administration to the host to protect the host against disease caused by the pathogen.

5       The invention further includes the use of the nucleotide vector or composition as a medicine. The invention additionally includes the use of the nucleotide vector or composition in the manufacture of a medicament for administration to a host for evoking an  
10       immune response to the at least one antigen.

Advantages of the present invention include:

- ease of administration;
- simplicity of construction; and
- an increased immune response to the antigen  
15       is provided.

#### BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following General Description and Examples with  
20       reference to the Figures, in which:

Figure 1 illustrates the cytotoxic T cell responses in mice immunized with different nucleoprotein gene encoding vectors;

Figure 2 shows the construction of plasmids for  
25       immunization; and

Figure 3, comprising panels a and b, shows the effect upon nucleoprotein-specific CTL responses following injection of co-linear B7-1 and B7-2 plasmids and co-injecting plasmids encoding GM-CSF and IL-12.  
30

#### GENERAL DESCRIPTION OF THE INVENTION

As described above, the present invention relates generally to nucleic acid immunization to produce an immune response (including a protective immune response)  
35       by administering nucleic acid molecules encoding antigens and co-stimulatory molecules, such a B7-1 and

B7-2, and, optionally, cytokines, such as GM-CSF, IL-12 and IL-4.

To demonstrate the impact of different co-stimulatory molecules and cytokines on immunogenicity, and particularly CTL induction, via nucleic acid immunization, a model antigen system was developed which was sub-optimal for CTL-induction to permit detection of increases in T-cell activation. NPV gene was cloned into NotI site of pRc/CMV vector (InVitrogen), and its orientation was determined by restriction digest analysis and sequencing. The NPO gene was amplified from EL4 cells, which had been infected with the influenzae strain X31, by RT-PCR, and was cloned into the pCR3 vector using TA Cloning Kit (InVitrogen). The sequence of the PCR primers for NPO were: NP-5': CGCGGCCGCCCCGCCATGGCGTCTCAAGGCACC (SEQ ID No: 15); NP-3': CGTCTAGATTATTAATTGTCGTA CTCTCTGC (SEQ ID No: 16). The two influenza nucleoprotein (NP) expression vectors (pCMV/NPv and pCMV/NPo) varied greatly in their ability to induce CTL responses (see Figure 1).

The pCMV/NPv vector, which encodes a native A/PR/8/34 NP, was better at inducing a CTL response than pCMV/NPo, which expresses a variant NP containing three mutations near the carboxy-terminus of the molecule (ref. 17). Whereas the pCMV/NPv plasmid induced an influenza NP 147-155 peptide-specific CTL response indistinguishable from that obtained from influenza-infected mice, the pCMV/NPo plasmid was unable to induce a CTL response above the pCMV vector control (Fig. 1).

In addition to their coding region differences, pCMV/NPv and pCMV/NPo also differ in their 5'- and 3'-untranslated regions (UTR), in that the former retained 5'- and 3'- sequences from the influenza virus UTR, whereas the latter did not. The entire NP coding region

and up to 100 nucleotides in the 5'- and 3'- flanking regions were sequenced for both pCMV/NPv and pCMV/NPo, and the following differences found. The pCMV/NPv NP DNA sequence contained two silent mutations, resulting  
5 in an amino acid sequence identical to that of A/PR/8/34 (Genbank accession #V01084), whereas pCMV/NPo had three nucleotide mutations leading to amino acid changes F304L, N370S, and G441R. The pCMV/NPv expression vector contained an influenza virus UTR upstream from the  
10 translation initiation methionine codon AGATAATCACTCACTGAGTGACATCAAAATC (Seq ID No: 1) and downstream of the stop codon, AGAAAAATACCCCTTGTTTCTACT (Seq ID No: 2) whereas pCMV/NPo lacked any viral UTR sequences.

15 When transfected into COS-7 cells, metabolically labelled with <sup>35</sup>S-methionine, and immunoprecipitated with an anti-NP monoclonal antibody, each expressed an intact NP molecule of the same molecular weight as that seen in influenza-infected cells, although the level of  
20 expression for pCMV/NPo was only about 20% of that seen with pCMV/NPv. However, when pCMV/NPo was transfected into P815, CTL target structure was formed which could be recognized by influenza-specific CTL. The differences in CTL responses are unrelated to the  
25 K<sup>d</sup>-restricted epitope NP aa 147 to 155, which is identical in each case. The pCMV/NPo vector provided a sub-optimal immunization plasmid which could be used to demonstrate the enhancement of the immunogenicity of an immunogen of the present invention.

30 In a preferred embodiment, co-stimulatory molecules are expressed on the same cells displaying the non-self, class I MHC-restricted epitope engaged by the precursor CTL. Co-linear plasmid immunization vectors were constructed for the simultaneous expression of B7-1 or  
35 B7-2 in the context of NPo (see Fig. 2). Fluorescence-activated flow cytometry on transiently

transfected COS cells was used in each case to establish that B7-1 or B7-2 was expressed at comparable levels on the surface of the relevant transfectants. In addition, the B7-1 and B7-2 gene products were confirmed to be functional using an *in vitro* co-stimulation assay. Vectors were also constructed for (a) the expression of GM-CSF, (b) the co-expression of the p35 and the p40 subunits of IL-12 in tandem, and (c) expression of the GM-CSF and IL-12 subunits on the same vector (Fig.2). Enzyme-linked immunosorbent assays and a bioassay were used to confirm the expression of the cytokines.

To confirm that constructs were functional, they were assayed through transient transfections into COS-7 cells. 48 hours following transfection, cells transfected with B7-1 or B7-2 containing constructs were stained for surface expression of the respective molecules, using the anti-B7-1 MAb 1G10, or the anti-B7-2 MAb GL1 (Pharmingen, San Diego, CA). The samples were analyzed on a FACScan flow cytometer (Becton Dickinson). The supernatants from GM-CSF and/or IL-12 transfected cells were tested for the presence of cytokine five days post-transfection. GM-CSF was detected using a mouse GM-CSF ELISA kit (Endogen, Cambridge, MA). In order to test for functional IL-12, a bioassay was used which is based on the ability of IL-12 to induce IFN $\gamma$  production in splenocytes. Supernatants from IL-12 transfected COS-7 cells were added to  $1 \times 10^7$  mouse splenocytes in serial dilutions (with the addition of 50U of human rIL2/mL). After a 48 hour incubation, the supernatants were tested for the presence of IFN $\gamma$ , using a mouse IFN $\gamma$  ELISA kit (Endogen).

The influence of the co-expression of sub-optimal NP antigen with different combinations of co-stimulatory molecules and cytokines was assessed by determining the NP-specific CTL response for sets of mice immunized in

parallel and then compared with influenza virus infected controls. After the first administration of plasmid, mice received booster injections at three and six weeks.

CTL activity was measured two weeks after each booster injection. In order to compare CTL responses from the different groups of mice, the data were normalized to the CTL response observed with spleen cells from influenza virus infected mice, the positive control included in each assay.

Addressing first the issue of adding a co-stimulatory molecule, CTL responses in mice were compared by immunizing with co-linear constructs containing NPo and either B7-1 or B7-2. The results indicate that co-expression of B7-1 fails to improve the low level of CTL activity seen with the sub-optimal NPo alone (Fig. 3). This is true after both the first boost (Fig. 3A) and the second boost (Fig. 3B) with NPo/B7-1. However, the co-linear expression of B7-2 in the NPo/B7-2 construct considerably enhances the post-first boost CTL response to the NP epitope. This effect is somewhat diminished after the second boost, suggesting that B7-2 might be better able to initiate than sustain the observed CTL response. These results are consistent with recent reports suggesting that the co-stimulatory activity of B7-2 may dominate that of B7-1 early in the initiation of T-cell responses (ref. 17).

Concerning the co-injection of plasmids expressing GM-CSF or IL-12, the cytokine-producing plasmids in the absence of any antigen-containing plasmid, do not greatly elevate the background NP-specific CTL response (Fig. 3). Even after a second boost with 100µg of the cytokine encoding plasmids, the level of lysis does not exceed 20% of the influenza virus control. When the NPo plasmid was co-injected with either the GM-CSF or the IL-12 plasmid, no enhancement of the NP specific CTL response was observed after the first boost (Fig. 3A).

However, after a second boost, anti-NP CTL responses were dramatically increased with both the GM-CSF and IL-12 constructs (Fig. 3B). When the co-linear plasmid expressing both GM-CSF and IL-12 was injected along with the NPo plasmid, the marked enhancement in the anti-NP CTL response was now seen after the first boost, and this high level of CTL activity remained essentially unchanged after the second boost. Thus, either GM-CSF or IL-12 can independently enhance the antigen specific CTL response, given time and multiple injections, but the combination of both cytokines appears to act synergistically to promote an earlier antigen specific CTL response. These data indicate the considerable positive impact that co-injection of the cytokine plasmids can have on the ability of antigens including a sub-optimal antigen to prime for an effective CTL response. They also indicate that the particular mix of co-injected cytokine expression units can have an influence on both the rate of development of the CTL response and its sustainability.

In order to demonstrate the effect of providing co-stimulatory molecules in the same cells expressing the antigen, while also altering the local cytokine environment, different combinations of the plasmids were injected and the NP-specific CTL responses measured. Although the NPo/B7-1 immunizations benefited marginally from the co-injection of either the GM-CSF or IL-12 plasmids after the first boost, a substantial increase in CTL activity was observed with the co-expressing GM-CSF/IL-12 plasmids (Fig. 3A). Because this same increase was also observed when the NPo plasmid was co-injected with GM-CSF/IL-12, this enhancement is unlikely to be attributed to a positive influence from the presence of B7-1. Likewise the pattern of responses after the second boost is very similar to that observed when these cytokine plasmids were used with NPo only,



indicating that there is little evidence for the involvement of B7-1 in responses to the co-linear NPo/B7-1 plasmid. In contrast, immunizations with the NPo/B7-2 co-linear plasmid mixed with either the GM-CSF, IL-12, or combination GM-CSF/IL-12 expressing plasmids resulted in elevated post first boost CTL responses (Fig. 3A) when compared to that seen with NPo alone. These responses diminished after the second boost, as did the response to the NPo/B7-2 plasmid alone, but in each case the benefit of the co-injected cytokine plasmid was clear (Fig. 3B). Thus, NP epitopes generated in the context of B7-2 co-stimulation are better able to take advantage of the GM-CSF and/or IL-12 in their immediate environment to produce a stronger CTL response.

The implications of being able to manipulate the magnitude and/or qualitative features of the immune response by nucleic acid immunization as provided herein are considerable. Antigens to which a cytotoxic T cell response is desired may be of poor immunogenicity. For example, tumour associated antigens, which may differ from self antigens by as little as single point mutations (ref. 19) can be expected to be poorly immunogenic. Because CTL responses to defined tumour associated antigens are known to be of therapeutic benefit (ref. 20), this situation represents a clear example where the augmentation of *in vivo* CTL responses to weak, but defined CTL determinants may be of significant benefit. A further practical advantage is the reduction in the amount of plasmid DNA required to be injected for even the strongest antigens to invoke a beneficial immune response. This may be particularly important with respect to safety concerns about the risk of plasmid DNA integration into genomic DNA, and the induction of anti-DNA antibodies (ref. 21).

The most appropriate combination of co-stimulatory

molecules and cytokines for a particular plasmid immunogen may vary depending on the type of immune response desired. For example, the nature of the co-stimulatory molecule (i.e. B7-1 or B7-2) can influence the  $T_H1$  versus  $T_H2$  balance of the induced response (ref. 10). Likewise the presence of certain cytokines during the initiation of a T cell response can have a dramatic impact on the immunological direction of a particular response. For example, IL-12 is known to promote the development of CTL responses and the  $T_H1$  pathway, whereas IL-4 greatly favours  $T_H2$ -governed responses (ref. 22).

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, and the treatment of infectious and neoplastic disease. A further non-limiting discussion of such uses is further presented below.

#### 1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the vectors as disclosed herein. The vaccine elicits an immune response in a subject. Immunogenic compositions, including vaccines, containing the nucleic acid may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 93/24640, (ref. 23)) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions, such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100%

of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment.

5       Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection  
10       facilitating agents, may advantageously be used.

      Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a  
15       phospholipid/glycolipid/polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

20       U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactide-co-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide),  
25       poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

      Published PCT application WO 91/06282 describes a  
30       delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery  
35       vehicle may additionally contain an absorption enhancer.

      The vectors described herein may be mixed with

pharmaceutically acceptable excipients which are compatible therein. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines, comprising the vectors described herein, may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intravenously, intradermally or intramuscularly, possibly following pre-treatment of the injection site with a local anesthetic.

Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Published PCT application WO 93/19183 (which is incorporated herein by reference thereto) describes the immunization of chicks against influenza by administration of DNA vectors encoding the influenza virus hemagglutinin type 7 (H7) gene. The vectors were administered intraperitoneally (ip), intravenously (iv), subcutaneously (sc), intranasally (in), intramuscularly (im), or intradermally (id). The immunogenic composition may be administered to mucosal surfaces by, for example the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed excipients, such as for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, capsules or sustained release formulations and may contain about 1 to 95% of the vectors described

herein.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual to synthesize the encoded antigen and mount an immune response. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 ng to about 1 mg of the vectors. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, genes encoding antigens from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and

stimulate such cells to elicit immune responses.

In particular embodiments of the present invention, the vector comprising a first nucleotide sequence encoding at least one antigen and a second nucleic acid molecule encoding at least one co-stimulatory molecule may be delivered in conjunction with a targeting molecule to target the vector to selected cells including cells of the immune system.

The polynucleotide vectors of the invention may be delivered to the host by a variety of procedures including injection, scarification, mucosal (for example intranasal) administration, Tang et al. (ref. 24) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 25) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

20

#### EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, immunology and fermentation technology used but not explicitly described in the disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those

skilled in the art.

#### Example 1

5 This Example describes CTL responses induced by immunization with different influenza NP-containing vectors.

Balb/c mice were immunized with 100µg of pCMV/NPv, pCMV/NPo or vector control pCMV, followed by boosting with 100µg of the same, plasmid 3 and 6 weeks later.

10 Spleen cells were obtained and the percent specific lysis was determined in a 4-hour <sup>51</sup>Cr-release. Spleens were harvested 2 weeks after the second boost. Mice more than 4 weeks post-recovery from infection with influenza strain X-31 were used as positive controls (solid square).

15 Spleen cells from 2 mice in each group were pooled and restimulated for 7 days *in vitro* with NP peptide (147-155)-pulsed autologous spleen cells and assayed against NP (147-155)-pulsed P815 cells. Briefly, spleen cells from 2 mice in each group

20 immunized with DNA, or recovered from infection with X-31, were pooled and cultured at 37°C/5% CO<sub>2</sub> for 7 days in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin (100U/ml), streptomycin (100µg/ml), 2mM L-glutamine at 5X10<sup>6</sup>/ml in the presence of

25 2.5X10<sup>6</sup>/ml syngeneic spleen cell stimulators which had been irradiated and pulsed for 1 hour with the H-2K<sup>d</sup>-restricted epitope, NP (147-155) at 0.1µg/µl. Cell-mediated cytotoxicity was assayed against P815 cells pulsed with NP147-155 peptide and labelled with 100µCi

30 of Na<sup>51</sup>CrO<sub>4</sub> (Amersham). Target cells at 10<sup>4</sup> per well were incubated for 4h. in triplicate at 37°C with serial dilutions of effector cells. Plates were then spun briefly in a bench-top centrifuge and 50µl of supernatant removed for counting in a TopCount

35 scintillation counter (Canberra-Packard, Canada). Maximum and spontaneous release was determined from

wells that contained 2% Triton-X100 and medium alone, respectively. Specific lysis was calculated as (experimental  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release)/(maximum  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release) X 100%. The data are shown in Figure 1 representative of three repeat experiments. pCMV/NPv (solid circle), pCMV/NPo (solid triangle) or vector control pCMV (open circle), DNA. These results are discussed above.

### Example 2

This Example describes the construction of immunization plasmids.

All genes were isolated using reverse transcriptase PCR amplification.

The B7-1 and GM-CSF genes were amplified from mRNA isolated from LPS-stimulated M12.4.1 cells, and the B7-2 gene was amplified from LPS-stimulated 38C13 cells. The two IL12-encoding genes p35 and p40 were amplified from LPS-stimulated WEHI-3 cells (ATCC TIB 68). The NP gene was amplified from EL4 cells (ATCC TIB 39), which had been infected with the influenza strain X-31. The mRNAs were isolated using the Quick prep micro RNA purification kit (Pharmacia, Piscataway, NJ), and the First-strand cDNA synthesis kit (Pharmacia) was used to synthesize the cDNA.

The sequences of the PCR amplifiers were as follows: B7-1-5': TAT AGC GGC CGC TCC AAA GCA TCT GAA GCT ATG GCT (SEQ ID No: 3); B7-1-3': TAT AGG GCC CAC AGA GAA GAA CTA AAG GAA GAC (SEQ ID No: 4); B7-2-5': TAT AGC GGC CGC GTT CCA GAA CTT ACG GAA (SEQ ID No: 5); B7-2-3' TAT AGG GCC CAC TGA ACA GTT CTG TGA CAT (SEQ ID No: 6); GM-CSF-5': TAT AGC GGC CGC CTC AGA GAG AAA GGC TAA GGT (SEQ ID No: 7); GM-CSF-3': TAT AGG GCC CTA TCT CTC GTT TGT CTT CCG (SEQ ID No: 8); P35-5': TAT GCG GCC GCG GTC CAG CAT GTG TCA ATC ACG (SEQ ID No: 9); P35-3': TAT GGG



CCC CCT TGA GCT TTC AGG CGG AGC (SEQ ID No: 10); P40-5':  
TAT GCG GCC AAG ATG TGT CCT CAG AAG CTA (SEQ ID No: 11);  
P40-3': TAT GGG CCC GTT GCA TCC TAG GAT CGG ACC (SEQ ID  
No: 12); NP-5': CGC GGC CGC CCG CCA TGG CGT CTC AAG GCA  
5 CC (SEQ ID No: 13); NP-3': CGT CTA GAT TAT TAA TTG TCG  
TAC TCC TCT GC (SEQ ID No: 14). All primers contain  
restriction enzyme sites in the 5' end, allowing for  
directional cloning (NotI and XbaI/ApaI).

The PCR products were initially ligated into the  
10 expression vector pcDNA3 (Invitrogen, San Diego, CA),  
sequenced and tested for expression by various *in vitro*  
assays (Fig. 2a). In order to confirm that our  
constructs were functional, they were assayed through  
transient transfections into COS-7 cells. 48 hours  
15 following transfection, cells transfected with B7-1 or  
B7-2 containing constructs were stained for surface  
expression of these respective molecules, using the  
anti-B7-1 MAb 1G10, or the anti-B7-2 MAb GL1  
(Pharmingen, San Diego, CA). The samples were analyzed  
20 on a FACScan flow cytometer (Becton Dickinson).

The supernatants from GM-CSF and/or IL-12  
transfected cells were tested for the presence of  
cytokine five days post transfection. GM-CSF was  
detected using a mouse GM-CSF ELISA kit (Endogen,  
25 Cambridge, MA). In order to test for functional IL-12,  
a bioassay was designed which is based on the ability of  
IL-12 to induce IFN $\gamma$  production in splenocytes.  
Briefly, supernatants from IL-12 transfected COS-7 cells  
were added to  $1 \times 10^7$  mouse splenocytes in serial  
30 dilutions (with the addition of 50U of human rIL2/ml).  
After a 48 hour incubation, the supernatants were tested  
for the presence of IFN $\gamma$ , using a mouse IFN $\gamma$  ELISA kit  
(Endogen).

In order to facilitate the cloning and expression  
35 of several genes in a single vector, the pGCVII plasmid  
(5 Prime-3 Prime Inc., Boulder, CO) was used as the

backbone for the immunization vectors. Expression cassettes, containing the gene of interest preceded by the cytomegalovirus (CMV) promoter and followed by the bovine growth hormone (BGH) polyadenylation signal, were subsequently removed from the pcDNA3 vector and inserted into the pGCVII plasmid (Fig. 2b). B7-1 and B7-2 cassettes were removed from BgIII/NaeI digestion and ligated into the BamHI/NaeI sites of plasmid pGCVII (to provide plasmids pGCV.7-1 and pGCV.7-2). The NP expression cassette was removed with an NruI/PvuII digest, and inserted into the PmeI sites of the plasmid pGCVII, pGCV.7-1 and pGCV.7-2 vectors (to provide plasmids pGCV.NPo, pGCV.NPo/7-1 and pGCV.NPo/7-2 respectively). The GM-CSF cassette was liberated by ScaI and PvuII digestion, and inserted into MscI site of plasmid pGCVII (to produce plasmids pGCV.GM-CSF).

Initially the p35 and p40 genes were cloned into the pcDNA3 vector separately. The p35 expression cassette was then removed by an NruI/PvuII digest and inserted into the NruI site of the p40 plasmid. The p35 and p40 expression units were finally excised on a single fragment by ScaI and DraIII digestion and inserted into either the MscI site of plasmid pGCVII (to provide plasmid pGCV.IL12) or the PmeI site of the pGCV.GM-CSF plasmid (to provide plasmid pGCV.GM/IL12).

Restriction maps for the respective vectors are shown in Figure 2.

### Example 3

This Example describes the NP-specific CTC response in mice immunized with nucleic acid molecules encoding NP, B7-1, B7-2, GM-CSF and IL-12 using the vectors described in Example 2.

Mice were injected with either 100µg of the NPo vector pGCV.NPo alone, or 100µg of the plasmid in which NPo was co-linear with B7-1 (plasmid pGCV.NPo/B7-1) or

5 B7-2 (plasmid pGCV.NPo/B7-2). Each plasmid was also co-  
injected with 100µg of the plasmids encoding GM-CSF  
(pGCV.GM-CSF), IL-12 (pGCV.IL12), or the GM-CSF/IL-12  
10 co-linear construct (pGCV.GM/IL12) in the hind leg  
muscle on 0, 3 and 6 weeks. Splenocytes were harvested  
2 weeks after the first boost (Fig. 3, panel a) or 2  
weeks after the second boost (Fig. 3, panel b) and  
assayed for NP-specific CTL activity. Effector  
15 populations were incubated for 4 hours at different  
effector to target ratios with <sup>51</sup>Cr-labelled P815 cells  
which had been pulsed with the NP (aa 147 to 155)  
peptide. Data normalized with respect to the influenza  
specific CTL response in each assay are depicted for the  
20 50:1 effector to target (E:T) ratio, where percent  
influenza control lysis is calculated as (percent  
specific lysis obtained with splenocytes from plasmid  
immunized mice)/(percent specific lysis obtained with  
splenocytes from X-31-primed mice) X 100 for each  
experiment. Lysis of labelled P815 in the absence of NP  
(147-155) was <10% at all effector to target ratios.  
The data shown in Figures 3a and 3b are representative  
results obtained in one set of mice from two independent  
repeats of this experiment and are discussed in detail  
above.

25

#### SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present  
invention provides certain vectors and methods for  
generating immune responses by co-administration of  
30 nucleotide vectors encoding antigens and co-stimulatory  
molecules. The immunization may also include  
administration of at least one cytokine-encoding gene.  
Modifications are possible within the scope of this  
invention.

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CLAIMS

What we claim is:

1. A nucleotide vector comprising:
  - (a) a first portion having a sequence encoding at least one antigen;
  - (b) a second portion having a sequence encoding at least one co-stimulatory molecule; and
  - (c) a promoter operatively coupled to each of said first and second portions for expression of said at least one antigen and said at least one co-stimulatory molecule.
2. The vector of claim 1 wherein the antigen is an antigen from a pathogen.
3. The vector of claim 2 wherein the pathogen is selected from the group consisting of viruses, bacteria and parasites.
4. The vector of claim 3 wherein the pathogen is an influenza virus.
5. The vector of claim 4 wherein the antigen is selected from the group consisting of structural and non-structural influenza virus antigens.
6. The vector of claim 4 wherein the antigen is selected from the group consisting of haemagglutinin, neuraminidase, nucleoprotein, NS1 and NS2.
7. The vector of claim 1 wherein the antigen is a tumour-associated antigen.
8. The vector of claim 7 wherein the tumour-associated antigen is selected from the group consisting of carcinoembryonic antigen (CEA), mutated tumor suppressor genes, mutated oncogenes and idiotypic markers for tumors.
9. The vector of claim 1 wherein the promoter is an immediate early cytomegalovirus promoter.
10. The vector of claim 1 wherein the co-stimulatory molecule is selected from the group consisting of B7-1 and B7-2.

11. The vector of claim 1 further comprising a third portion having a sequence encoding at least one cytokine.
12. The vector of claim 11 wherein the at least one cytokine is selected from the group consisting of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12), interleukin-4 (IL-4) and fragments thereof retaining cytokine activity.
13. The vector of claim 2 or claim 11 formulated as a vaccine for in vivo administration to a host to protect said host against disease caused by said pathogen.
14. A composition comprising the vector of claim 1 and a second nucleotide vector comprising:
  - (a) a first portion having a sequence encoding at least one cytokine; and
  - (b) a promoter operatively coupled to said first portion of said second vector for expression of said cytokine.
15. The vector of claim 14 wherein the at least one cytokine is selected from the group consisting of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-12, interleukin-4 and fragments thereof retaining cytokine activity.
16. A method of generating an immune response in a host, comprising administering to the host an effective amount of a first nucleic acid molecule encoding at least one antigen, a second nucleic acid molecule encoding at least one co-stimulatory molecule and a promoter operatively coupled to each of said first and second nucleic acid molecules for expression of said at least one antigen and said at least one co-stimulatory molecule.
17. The method of claim 16 wherein said first nucleic acid molecule and said second nucleic acid molecule are contained in a single nucleotide vector.
18. The method of claim 17 wherein the antigen is an

antigen of a pathogen.

19. The method of claim 18 wherein the pathogen is selected from the group consisting of bacteria, viruses and parasites.

20. The method of claim 17 wherein the antigen is a tumour-associated antigen.

21. The method of claim 17 wherein the immune response is a cytotoxic T cell immune response.

22. The method of claim 18 wherein the immune response confers protection to the host against disease caused by the pathogen.

23. The method of claim 17 wherein the vector is administered intraperitoneally, intravenously, subcutaneously, intramuscularly, intradermally or to a mucosal surface of said host.

24. The method of claim 23 wherein the vector is administered intranasally.

25. The method of claim 17 further comprising the step of administering to the host at least one third nucleic acid molecule encoding at least one cytokine and a promoter operatively coupled to said third nucleic acid molecule for expression of said cytokine.

26. The method of claim 25 wherein the cytokine is selected from the group consisting of macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12), interleukin-4 (IL-4) and fragments thereof retaining cytokine activity.

27. The method of claim 25 wherein the at least one third nucleic acid molecule encoding the at least one cytokine is contained within the single nucleotide vector.

28. The method of claim 25 wherein the at least one third nucleic acid molecule encoding the at least one cytokine is contained within a second nucleotide vector.

29. A method of using a first gene encoding an antigen and a second gene encoding a co-stimulatory molecule to



produce an immune response in a host, comprising the steps of:

- (a) constructing a nucleotide vector comprising said first and second genes;
- (b) operatively coupling to each of said first and second genes a control sequence to direct expression of said first and second genes in said host; and
- (c) introducing said vector into the host.

30. A method for producing an immunogenic composition for evoking a specific immune response to an antigen in a host to which said immunogenic composition is administered, comprising the steps of:

- (a) constructing a nucleotide vector comprising a first gene encoding the antigen and a second gene encoding a co-stimulatory molecule;
- (b) operatively coupling to each of said first and second genes a control sequence to direct expression of said first and second genes in said host; and
- (c) formulating said vector as the immunogenic composition for *in vivo* administration to the host.

31. The method of claim 30, wherein the antigen is an antigen of a pathogen and the immunogenic composition is formulated as a vaccine for administration to the host, to protect the host against disease caused by the pathogen.

32. The use of a nucleotide vector as claimed in claim 1 or 11 or of a composition as claimed in claim 14 as a medicine.

33. The use of a nucleotide vector as claimed in claim 1 or 11 or of a composition as claimed in claim 14 in the manufacture of a medicament for administration to a host for evoking an immune response to the at least one antigen.

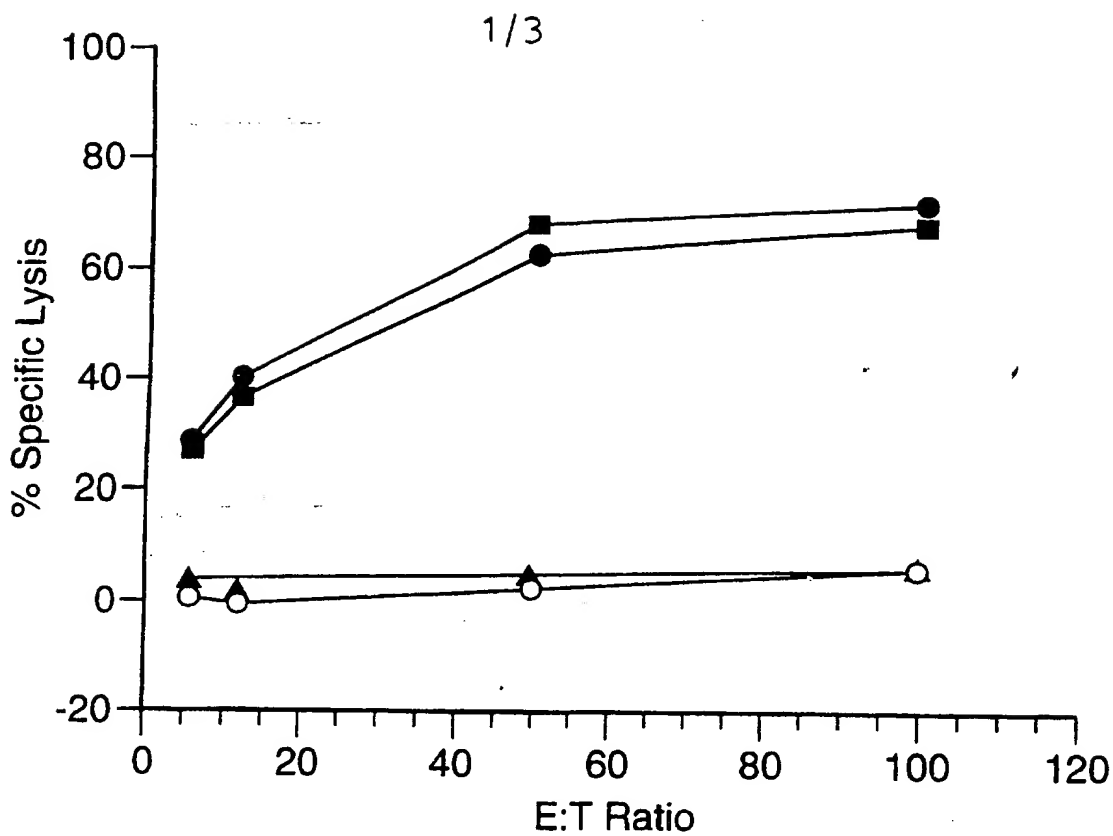


FIG.1

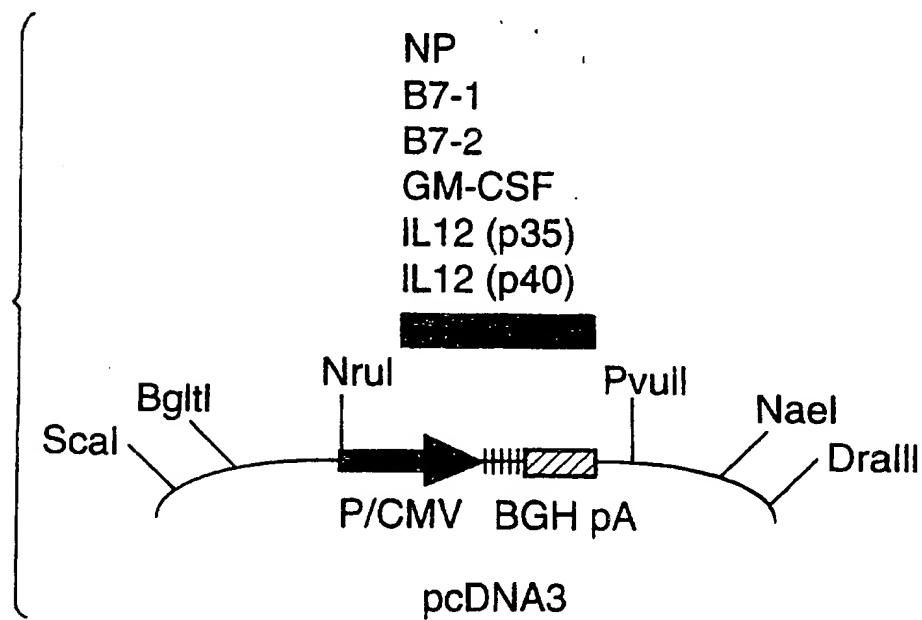


FIG.2A

2 / 3

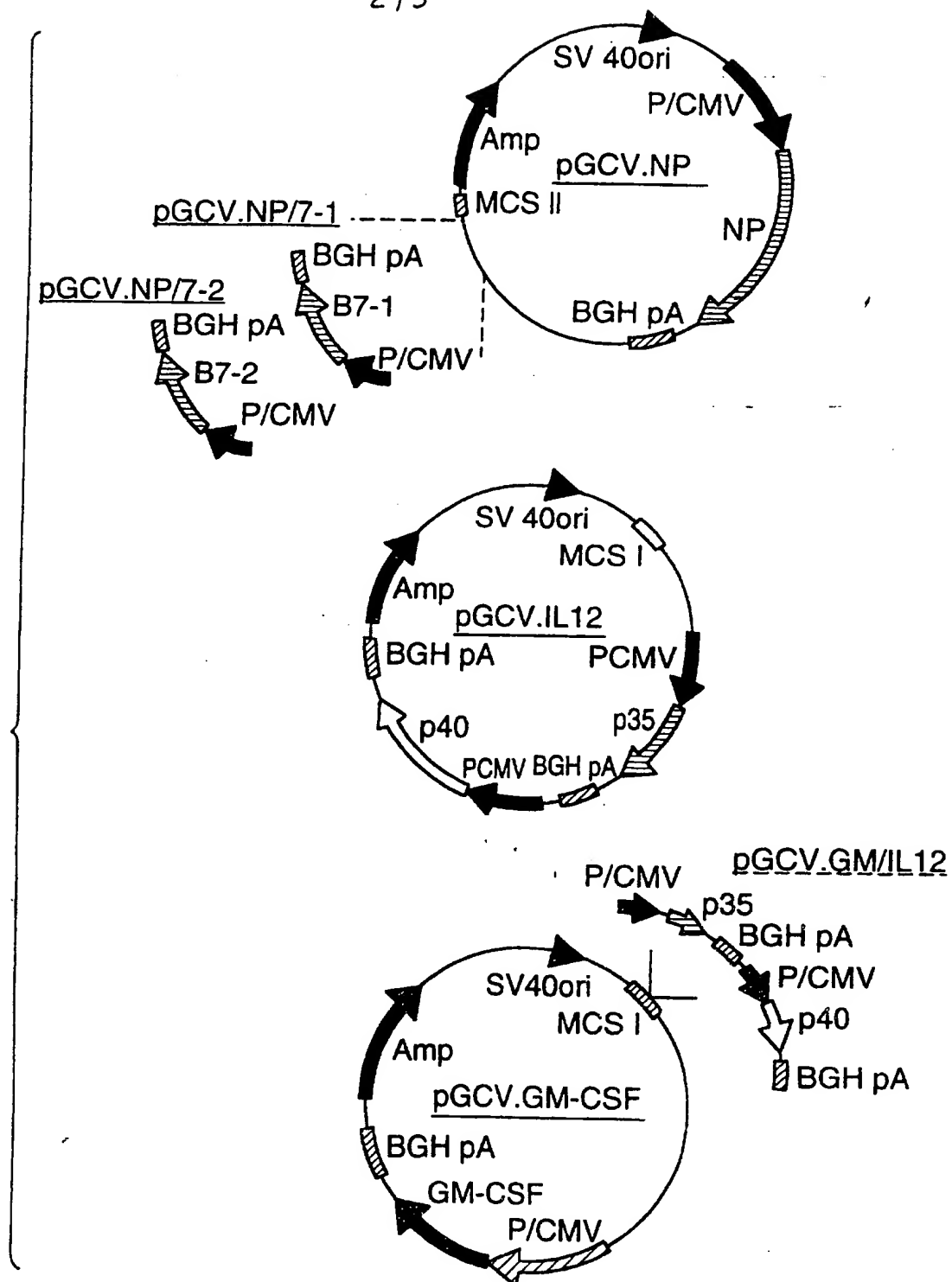


FIG.2B

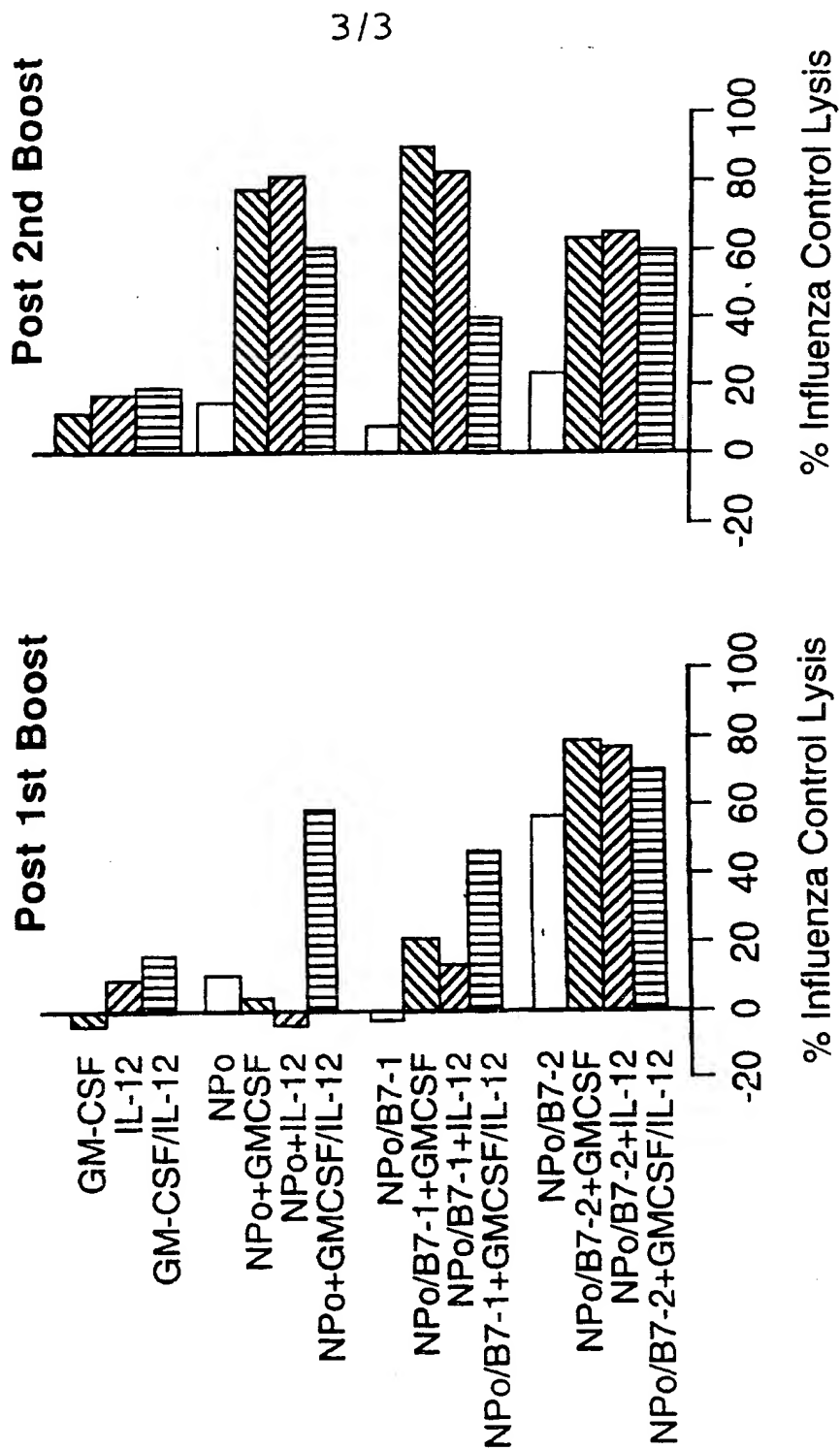


FIG.3B

FIG.3A

# INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/CA 97/00162

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 A61K48/00 A61K31/70 //C12N15/12, C12N15/19, C12N15/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE THERAPY, vol. 3, no. 1, January 1996, pages 67-74, XP002035090 CONRY R.M. ET AL.: "Selected strategies to augment polynucleotide immunization" see page 67 - page 72, left-hand column	1-3, 7-20, 22, 23, 25-33
Y	---	4-6, 21, 24
Y	SCIENCE, vol. 259, 19 March 1993, pages 1745-1749, XP002009751 ULMER J.B. ET AL.: "Heterologous Protection against influenza by injection of DNA encoding a viral protein" see abstract see page 1746, left-hand column, line 29, - page 1748 ---	4-6, 21
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

17 July 1997

Date of mailing of the international search report

30.07.97

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 97/00162

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 05853 A (UNIV CALIFORNIA ;CARSON DENNIS A (US); RAZ EYAL (US); HOWELL MERED) 2 March 1995 see page 52, line 10 - page 53, line 7; figures 3-5; example III	24
A	see page 63, line 17 - page 64, line 2; figure 14; example VII ---	4-6
X	TASCON R ET AL: "POLYNUCLEOTIDE VACCINATION INDUCES A SIGNIFICANT PROTECTIVE IMMUNE RESPONSE AGAINST MYCOBACTERIA" 1996 , VACCINES 96. MOLECULAR APPROACHES TO THE CONTROL OF INFECTIOUS DISEASES, PAGE(S) 45 - 49 , COLD SPRING HARBOR LABORATORY PRESS XP000673326 see the whole document ---	1-3,9, 10,13, 16,29-33 ,
A	HUMAN GENE THERAPY, vol. 7, no. 4, 1 March 1996, pages 525-529, XP002035091 CAYEUX S. ET AL.: "Coexpression of interleukin-4 and B7.1 in murine tumor cells leads to improved tumor rejection and vaccine effect compared to single gene transfectants and a classical adjuvant " see page 525 - page 526, left-hand column, line 13 see page 527, right-hand column, paragraph 4 - page 528, left-hand column ---	10,12, 15,26
A	CANCER RESEARCH, vol. 55, 1 November 1995, pages 4980-4987, XP002035092 COUGHLIN C.M. ET AL.: "B7-1 and interleukin 12 synergistically induce effective antitumor immunity" see page 4980 - page 4981, left-hand column, line 35 discussion see page 4985 - page 4986 ---	10,12, 15,26
A	IMMUNITY, vol. 2, no. 2, February 1995, pages 129-135, XP000670098 XIANG Z. ET AL: "Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines" cited in the application see the whole document ---	1-9, 13-24, 29-33

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 97/00162

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CANCER RESEARCH, vol. 54, 1 November 1994, pages 5552-5555, XP002035093 HODGE J.W. ET AL.: "Induction of antitumor immunity by recombinant Vaccinia Viruses expressing B7-1 or B7-2 costimulatory molecules" see abstract see page 5554; figure 2</p> <p style="text-align: center;">---</p>	10
P,X	<p>MOLECULAR MEDICINE, vol. 2, no. 5, September 1996, pages 545-555, XP002035094 BUELER H. AND MULLIGAN R.C.: "Induction of antigen-specific tumor immunity by genetic and cellular vaccines against MAGE: enhanced tumor protection by coexpression of Granulocyte-Macrophage Colony-Stimulating Factor and B7-1" see page 545 - page 546, left-hand column see page 547, right-hand column, paragraph 2 - page 548, right-hand column see page 549, right-hand column, line 6 - page 553, left-hand column</p> <p style="text-align: center;">---</p>	1,7,9, 10,16, 20,21, 29,30, 32,33
T	<p>THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 10, 15 May 1997, pages 4591-4601, XP002035095 IWASAKI A. ET AL.: "Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines"</p> <p style="text-align: center;">-----</p>	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 97/ 00162

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16-29, 32  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 32 as far as in vivo methods are concerned, and claims 16-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



FURTHER INFORMATION C NTINUED FROM PCT/ISA/210

- 1) claims 1-10, 16-24, 29-31 all totally; claims 11-15, 25-28, 32, 33 all partially.

Nucleotide vector comprising a sequence coding for an antigen and a sequence coding for a co-stimulatory molecule. Uses as vaccine.

- 2) claims 11-15, 25-28, 32, 33 all partially.

Nucleotide vector(s) comprising a sequence coding for an antigen, a sequence coding for a co-stimulatory molecule and a sequence coding for a cytokine. Uses as vaccine.

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International Application No.

PCT/CA 97/00162

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505853 A	02-03-95	AU 7639194 A	21-03-95
		EP 0714308 A	05-06-96
		JP 9501936 T	25-02-97
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